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X inactivation and reactivation in X-linked diseases

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ABSTRACT

X chromosome inactivation (XCI) is the phenomenon by which mammals compensate for dosage of X-linked genes in females (XX) *versus* males (XY). XCI patterns can be random or show extreme skewing, and can modify the mode of inheritance of X-driven phenotypes, which contributes to the variability of human pathologies. Recent findings have shown reversibility of the XCI process, which has opened new avenues in the approaches used for the treatment of X-linked diseases.

Contents

1. Introduction

Mammalian somatic cells can determine how many X chromosomes are present, in order to transcriptionally silence all but one X chromosome per set of autosomes. This phenomenon is known as X chromosome inactivation (XCI), and it compensates for dosage of X-linked genes in females (XX) *versus* males (XY). Once established, XCI is stably maintained through epigenetic remodeling, and it is mitotically transmitted, such that each cell will contain both one active and one inactive X chromosome (*i.e.*, Xa, Xi, respectively). The maternal and paternal X chromosomes (*i.e.*, Xm, Xp, respectively) faithfully show the same probability for inactivation, and this randomness in the choice phase that occurs early in embryogenesis gives rise to the overall 1:1 ratio of cells that express either the Xm or the Xp.

However, there is a wide range of variations from this ratio (*i.e.*, the XCI pattern) in the normal female population [1], and the same X chromosome can be preferentially inactivated in most of the cells (*i.e.*, skewed, or unbalanced, XCI). An unbalanced XCI pattern can be achieved through clonal selection of cells after the initial XCI is correctly based upon random choice. Alternatively, skewing of XCI can be influenced genetically, which means that the primary XCI is not established at random [1]. Genetic/genomic elements that can propel this selection bias have been defined in the mouse X-controlling element [2,3]. Furthermore, imprinted inactivation of Xp occurs physiologically in the murine trophoblast [4], although the underlying mechanisms remain to be explored.

Abbreviations: iPSCs, induced pluripotent stem cells; MeCP2, methyl CpG binding protein 2; RTT, Rett syndrome; Xa, active X chromosome; XCI, X chromosome inactivation; Xi, inactive X chromosome; XIST, X-inactive specific transcript; Xm, maternal X chromosome; Xp, paternal X chromosome.

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In humans, it is not completely clear whether or not the XCI choice is genetically driven (see also Peeters et al. in this issue, page 71). Interestingly, XCI skewing appears to be a heritable trait, which suggests that one or more X-linked genes can influence the XCI patterns [5–7]. Moreover, the distribution of XCI patterns in female populations is more consistent with a mathematical model of genetically influenced choice [8]. This choice appears to be a complex hallmark that is influenced by genetic factors (both autosomal and X-linked) and/or parent-of-origin effects, as has already been suggested for mice [9].

In recent years, it has become clear that XCI is a more complex process than initially believed, with the XCI ratio showing variations during aging in healthy females, which influences the expression of a higher number of phenotypes, even under physiological conditions (*i.e.*, aging hematopoiesis [10]). Of note, as well as inter-individual variability in XCI patterns, intra-individual variability has also been documented, even with differences at the cell-type level [11]. The complexity of the overall phenomenon also arises as some X-linked genes can partially escape XCI. These genes have usually been defined as ones that show 10% expression from the Xi allele, compared to the Xa allele [12], and they have been estimated to represent 15% of the human X-linked genes [13] or a little more [14], although at least constitutive 12 and many more cell-type specific escapee genes have been identified in mice to date [15]. The most paradigmatic escapee gene is XIST (X-inactive specific transcript), which is expressed only by Xi [16]. Xi escapee genes have been proposed to be causative candidates of Turner (XO) and Klinefelter (XXY) syndromes, as in such conditions their dosage is lower and higher, respectively, than in euploid cells [12]. Moreover, the high intraspecific variability in escapee gene expression might be responsible for the variability in phenotypic severity between individuals with the same polyX karyotype [14].

Since its discovery in the 1960s, XCI has held the attention of researchers operating in different fields. Indeed, XCI patterns can modify the classical mode of inheritance of X-driven phenotypes [17], which contribute to the variability. It has been shown that heterozygous female carriers of an X-linked dominant disorder can be phenotypically healthy, as the normal X chromosome is active in nearly all somatic cells.

On the contrary, unbalanced XCI can functionally transform an X-linked recessive trait into a dominant trait, if the X chromosome with the normal allele has been turned-off in a greater proportion of cells. Moreover, an X chromosome that carries structural rearrangements such as deletions and duplications is often inactive [18]. In contrast, in the case of X-autosomal translocations, the correct gene dosage of the translocated autosomal segment is ensured, with the normal X chromosome being inactive in the presence of balanced translocations, or active in the unbalanced situation [19]. These patterns are usually correlated with a normal or mild phenotype, although discrepancies between XCI ratios and severity of phenotype have also been reported [18,20].

Here, we provide a brief overview of the methods that have been adopted to assess XCI, while illustrating some of the technical limitations and the recent cellular tools that have proven promising to overcome such restrictions. Then, we discuss how XCI patterns can influence the phenotypic variability in X-linked disorders, with a comparison of the results obtained in human and mouse models. Finally, we report how those involved in the field of XCI can guide the new therapeutic approaches, particularly in the treatment of X-linked disorders. Indeed, even if XCI is tightly controlled, recent findings have shown reversibility of this process in differentiated cells, with promising preliminary data obtained relating to the treatment of Rett syndrome (RTT, MIM #312750). Spurred on by these outcomes, RTT is used here as a clarifying model to promote the aims of the present review.

2. Techniques to evaluate XCI status

The mechanisms behind the establishment and maintenance of XCI have been deeply investigated [21], which has improved our knowledge of this process. The methods that are used to quantitatively define XCI *status* in humans have been conserved over time, and these are essentially based on: (i) differential DNA methylation of X alleles; (ii) expressed polymorphisms; (iii) analysis of DNA replication timing. Sometimes these approaches have been combined [22,23], although methylation-based assays are much more widespread because DNA is more easily accessible and is more stable than RNA. Also, promoter regions of X-linked genes on Xi are generally hypermethylated, to maintain their inactive state [24].

Among the DNA-methylation-based approaches, the most accepted and practiced assay is known as the human androgen receptor (HUMARA) assay (Fig. 1 A, upper panel). This is a methyl-CpG-sensitive restriction-endonuclease-based PCR assay that targets the polymorphic short tandem repeat of the Xq-linked androgen receptor (AR) gene. Importantly, the methylation *status* of the AR alleles on Xi correlates with the whole XCI [25]. According to current knowledge, as Xp and Xm have the same 50% probability of being methylated and inactivated, a 1:1 ratio for XCI is expected if it is a random event. All other values of the XCI ratio that are significantly different from this theoretical ratio reflect preferential selection of the X chromosome that is to be inactivated. Skewed, or extremely skewed, XCI is arbitrarily defined as the inactivation of the same X chromosome in 75% to 80%, or 90% to 95%, of cells, respectively [26]. In 1999, a more rapid and accurate methylation-specific PCR assay was developed (Fig. 1A, lower panel), which is independent of the use of methylation-sensitive enzymes [27]. This is a two-step approach, where PCR is performed with primers specific for methylated *versus* unmethylated DNA, following the chemical modification of DNA with sodium bisulfite. The sodium bisulfite treatment converts the methylation difference into a DNA sequence difference, as unmethylated cytosines, but not methylated ones, are converted into uracil. The HUMARA assay is, however, still used in current diagnosis, although its combination with PCR primer sets derived from other polymorphic regions of the X chromosome can increase the informative females for XCI pattern studies to 96% [28,29].

One of the latest loci to be discovered as informative in such XCI studies is the retinitis pigmentosa 2 (RP2) gene [30]. Unlike the AR repeat, the RP2 repeat has not been associated to pathological conditions to date, and it has been evolutionarily conserved in nonhuman primates. As opposed to the AR repeat, the RP2 gene is located on the short arm of the X chromosome. Therefore, a combined AR/RP2-based assay is expected to be more efficient for the analysis of XCI. Independent of the gene, this kind of assay is still restricted to easily available blood cells or oral mucosal cells, and it takes into account only one of the molecular mechanisms that sustains XCI (*i.e.*, DNA methylation).

Alternatively, the XCI *status* can be measured at the RNA level using expressed polymorphisms of X-linked informative genes (*e.g.*, XIST [31]), which can be identified through database searches [32]. For example, this method was successfully applied to the determination of the X and Y inactivation statuses of the PAR2 gene SYBL1 [33]. To avoid a false XCI ratio, additional factors that influence gene expression should also be considered. In addition to XCI itself, allele-specific gene transcription can be attributed to variants in *cis*-regulatory elements and/or to tissue-specific regulators. As a consequence, the higher the number of genes tested, the better the estimation of the XCI ratio achieved. Nowadays, RNA sequencing